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Route of TT Virus Infection in Children

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TT virus (TTV) is a novel viral agent, detected recently in non-A to E hepatitis cases. Little is known about its natural history or routes of transmission in childhood. For the detection of serum TTV DNA, semi-nested polymerase chain reaction (PCR) was carried out using TTV-specific primers and TTV nucleotide sequences were determined by the dideoxy chain-mediated termination method. Five of the 70 children studied (including 20 hepatitis B virus [HBV] carriers, 40 children born to HBV carrier mothers and 10 children born to hepatitis C virus [HCV] carrier mothers) had serum TTV DNA. Three of the 5 children had siblings (4 in total), so that a total of 9 children were studied to determine the time of initial serum TTV DNA detection. In the 8 seropositive children, the time of serum TTV DNA detection ranged from 6 to 14 months after birth, and TTV DNA persisted thereafter throughout the follow-up period. The TTV DNA-negative child was assessed most recently at 6 months of age. TTV DNA was detected in only 2 of the 4 mothers tested (families 2 and 3). When 271-bp TTV DNA fragments from each of the 8 children were sequenced, the degree of homology between siblings in families 1–3 was 100%, 99.5%, and 92.3%, respectively. The degree of homology between child-mother pairs of families 2 and 3 was 99.5–100% and 62.6–63.9%, respectively. The distribution of different TTV strains was consistent within families, except for family 3. None of the TTV-infected children had elevated levels of alanine aminotransferase or clinical signs of liver disease. ***J. Med. Virol. 59:204–207, 1999.***

KEY WORDS: novel viral agent; blood-transmittable virus; sequence analysis

INTRODUCTION

Recently, a novel viral agent designated TT virus (TTV) was identified [Nishizawa et al., 1997; Okamoto et al., 1998a]. TTV is present in the sera of about 50% of adult patients who have acute or chronic non-A to E hepatitis in Japan. TTV has also been found in patients with liver disorders in the UK (Naoumov et al., 1998] and North America [Charlton et al., 1998]. However, little is known about the epidemiology and clinical manifestations of TTV infection during childhood. Therefore, we evaluated the time of initial serum TTV DNA detection in children and investigated the presence of serum TTV DNA in the siblings and mothers of serum TTV DNA positive cases in an attempt to investigate the route of transmission of TTV in children.

METHOD

Sera from 70 Japanese children were assessed for the presence of TTV DNA. These 70 children consisted of 20 hepatitis B virus (HBV) carriers (mean age ± SD at assessment: 105 ± 58 months, range 12–179 months), 40 children (age 12–24 months) born to HBV carrier mothers and 10 children (age 12–24 months) born to hepatitis C virus (HCV) carrier mothers. Most children in the second group had been inoculated against perinatal transmission of HBV by injection of 1 ml of hepatitis B immunoglobulin (HBIG), once within 48 hr of birth or twice, within 48 hr and 2 months after birth. They received the hepatitis B vaccine (recombinant vaccine produced by Chinese hamster ovary cells) at 2, 3, and 5 months of age, as recommended by the Ministry of Health and Welfare of Japan. None of the children in the third group showed evidence of HCV infection during the follow-up period. Upon identification of TTV DNA positive children, the siblings and mothers were examined for the presence of TTV DNA in the serum.

Blood samples obtained at various points were separated immediately and the serum was stored at −30°C until use. Parental consent was obtained for blood sampling. Each serum sample (100 μl) was incubated at 65°C for 2 hr in the presence of proteinase K (150 μg/

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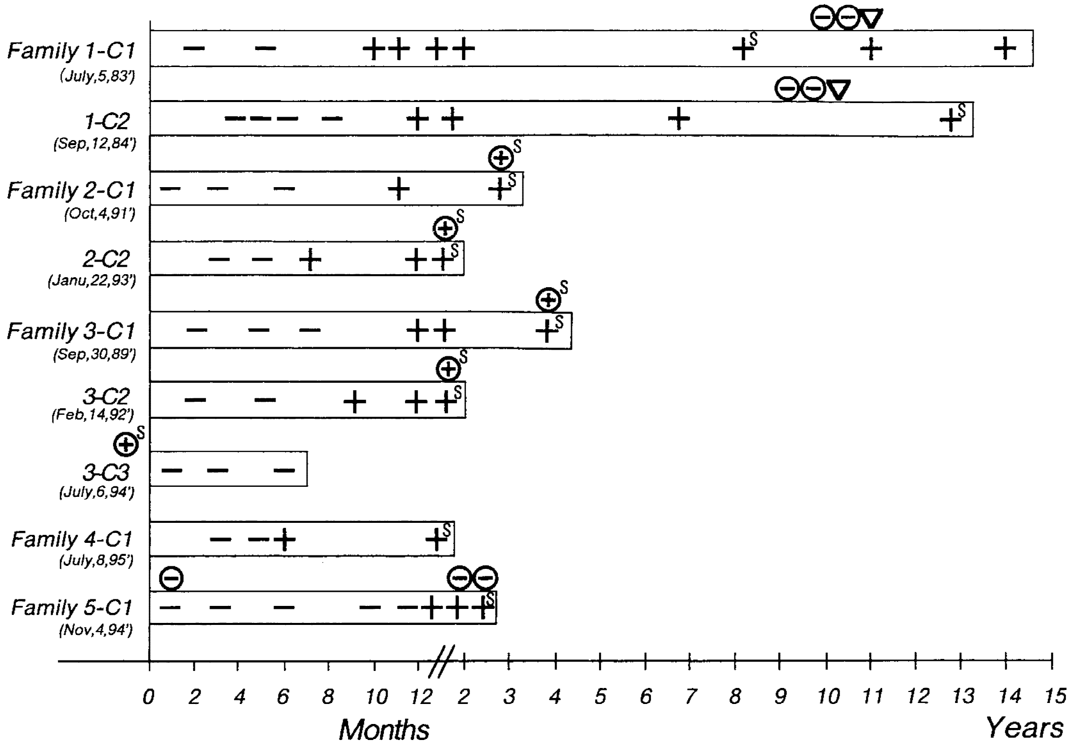

Fig. 1. Serum TT virus (TTV) DNA in the nine children. Birthday of each child shown in parentheses. C, child; +, serum TTV DNA positive; –, serum TTV DNA negative; ⊕, samples collected from mothers positive for serum TTV DNA. Sample of mother of family 3 collected at middle stage of her last pregnancy; ⊖, samples collected from mothers negative for serum TTV DNA. Samples of the mother in family 1 and 2 were tested two and three times, respectively; ∇, sample collected from father negative for serum TTV DNA negative; S, samples used for sequence analysis.

ml), 0.5% sodium dodecyl sulfate, 5 mM ethylene diamine tetra-acetic acid, and 10 mM Tris-HCl (pH 8.0). TTV DNA was extracted from the serum using the phenol-chloroform-isoamyl alcohol procedure described previously [Miyake et al., 1996]. Extracted DNA was precipitated with cold ethanol in the presence of carrier transfer RNA (5 μg) and was dissolved in 50 μl of 10 mM Tris-HCl (pH 8.0). Using 2-μl samples of each isolated DNA, semi-nested polymerase chain reaction (PCR) was performed with two sets of primers specific for TTV DNA [Okamoto et al., 1998a]. The sense primers were 5′-ACAGACAGAGGAGAAGGCAACATG-3′ (for first round PCR) and 5′-GGCAACATGTTATGGATAGACTGG-3′ (for second round PCR) and the antisense primer was 5′-CTGGCATTTTACCATTTCCAAAGTT-3′ (for first and second round PCR). First round PCR was performed in a 20-μl reaction volume containing 0.5 unit of Taq polymerase (Takara Shuzou, Otsu, Japan), 0.5 μM of each primer, 0.125 mM of each deoxyribonucleoside triphosphate, 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.3). Amplification was undertaken for 35 cycles consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C (with an additional 7-min extension in the last cycle). Second round PCR was carried out for 30 cycles under the same conditions using 2 μl of a 10×-diluted solution of the first-round PCR products. PCR products were separated by electrophoresis on 3% agarose gels, stained with ethidium bromide, then observed under ultraviolet light. Sera were identified as TTV DNA-positive if a 271-bp band was detected. All reagents were examined for TTV DNA contamination, and all experiments were performed with positive and negative control sera.

For sequence analysis, the amplified DNA fragments were purified using the glass powder system, EASYTRAP (Takara Shuzou, Otsu, Japan). The purified DNAs were sequenced by the dideoxy-mediated chain-termination method, using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a 373A fluorescent DNA sequencer (Model 373A, Applied Biosystems, Foster City, CA). In the sequencing reaction, primers for second round PCR were used as sequence templates. The sequences were compared by multiple sequence alignments and a phylogenetic tree was constructed using the unweighted pair-group method with arithmetic mean (UPGMA). Genetic distances were calculated using ODEN version 1.2 software for molecular evolutionary analysis [Nei and Gojobori, 1986; Ina, 1994].

RESULTS

Serum TTV DNA was detected in 1 (family 1) of the 20 HBV carriers, in 3 (families 2–4) of the 40 children born to HBV carrier mothers, and in 1 (family 5) of the 10 children born to HCV carrier mothers. Three of the 5 children (families 1–3) had siblings (1, 1, and 2, re-

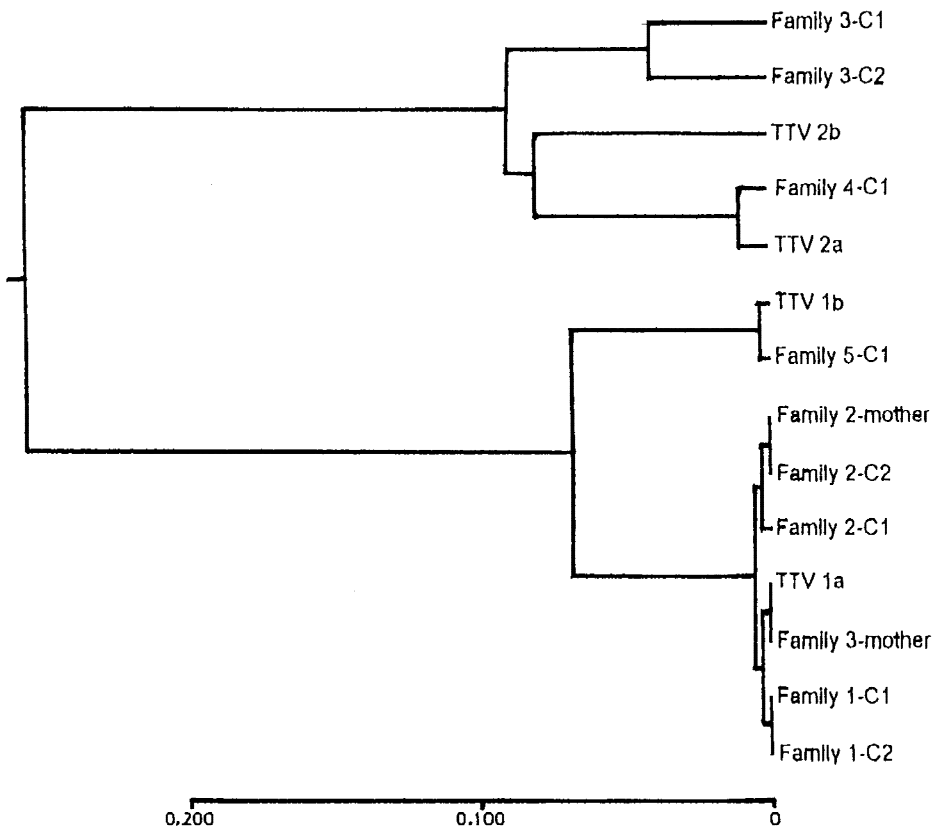

Fig. 2. Phylogenetic analysis. This tree was constructed by unweighted pair-group method with arithmetic mean. Horizontal axis shows number of nucleotide substitutions per site.

spectively) who were also positive for serum TTV DNA, except for 1 (family 3-C3) who was negative. Serum TTV DNA was detected in only 2 of the 4 mothers tested (mothers of families 2 and 3; mother of family 4 not tested). The father of family 1, the only one who agreed to be tested, was negative. The siblings were also inoculated against perinatal transmission of HBV by immunoprophylaxis that was judged to be successful. None of the mothers of the 5 serum TTV DNA-positive children had a history of blood transfusion, nor were any of them intravenous drug users.

The time of initial serum TTV DNA detection and the results of follow-up of TTV DNA seropositivity in the 9 children are shown in Figure 1. Serum TTV DNA in the 9 children was negative for several months after birth and was first detected in 8 of the 9 children between 6 and 14 months after birth (the last follow-up sample of the TTV DNA-negative child of family 3 was taken at 6 months of age), and the positivity persisted thereafter throughout the follow-up period. None of the TTV-infected children, including the child of family 1 (C1) who developed into a hepatitis B e antigen positive, asymptomatic HBV carrier, demonstrated elevation of serum alanine aminotransferase (ALT) levels (>40 IU/L).

A homology search for a 222-bp sequence [spanning nt 1959–2180 of the original N22 clone reported by Okamoto et al., 1998] was performed in each family. The degree of homology between siblings in families 1–3 was 100%, 99.5%, and 92.3%, respectively. The degree of homology between child–mother pairs of families 2 and 3 was 99.5–100% and 62.6–63.9%, respectively. All sequences were compared with TTV consensus sequences [subgroups 1a, 1b, 2a, and 2b reported by Okamoto et al., 1998] using the phylogenetic analysis method (Fig. 2). The TTV sequences of the children of family 1, the two children and mother of family 2, and the mother of family 3 belonged to subgroup 1a, whereas the child of family 4 and the child of family 5 belonged to subgroups 2a and 1b, respectively. The children of family 3 belonged to an external subgroup. These results revealed that the distribution of different TTV strains was consistent within families, except for family 3.

DISCUSSION

The prevalence of serum TTV DNA in the present study was 5/70 (7.1%). In addition, as shown in Figure 1, the results suggest that a long-term persistence of serum TTV occurs when the children become infected with TTV during infancy.

It is unclear whether the route of transmission of TTV in children without a history of blood transfusion is maternal, takes a nontransfusion-related horizontal route, or both. Consequently, the presence of serum TTV DNA was investigated in the siblings and mothers of serum TTV DNA-positive cases in an attempt to in-

vestigate the route of transmission of TTV. Although this study was retrospective, serum TTV DNA was detected in only 2 of the 4 mothers tested. TTV is believed to be a blood-transmittable virus [Nishizawa et al., 1997; Charlton et al., 1998; Naoumov et al., 1998; Okamoto et al., 1998a; Simmonds et al., 1998], but the levels of circulating virus in infected cases are relatively lower than those of HBV, HCV, and GBV-C/hepatitis G virus (HGV) [Simmonds et al., 1998]. Although the 7 serum TTV DNA-positive children tested in the present study were inoculated against perinatal transmission of HBV by immunoprophylaxis, there was a possibility that TTV infection occurred through HBIG injection. However, TTV DNA was not detected in samples from 7 commercially available HBIGs, nor from three different lots of HBIG produced by the same procedure as the HBIG used in the present cases. Furthermore, the 7.5% (3/40) serum TTV prevalence rate in the present cases did not differ significantly from the 5.1% (10/197) prevalence reported in children with no history of blood or blood product transfusion [Goto et al., 1999]. Thus, although such a possibility cannot be completely ruled out, the risk of TTV infection through HBIG injection is low.

Furthermore, although this study was retrospective, the results from families 1–3 in the present homology search for nucleotide sequences, which showed the same type of TTV in the siblings of each family, suggest that the route of TTV transmission was intrafamilial. In particular, the results of family 2 suggest that the route of TTV transmission was mother-to-infant transmission. On the other hand, sequence homology analysis revealed a difference between the TTV strains present in the mother and children of family 3. It appears that at least two children of family 3 were infected from an extrafamilial source of TTV. In a recent paper, Okamoto et al. [1998b] demonstrated the possibility of fecal transmission of TTV. Thus, a fecal-oral transmission route of TTV should be considered in cases with extrafamilial transmission of TTV.

To date, the pathogenetic role of TTV in liver disease remains unclear [Nishizawa et al., 1997; Charlton et al., 1998; Naoumov et al., 1998; Okamoto et al., 1998a; Simmonds et al., 1998]. In the present study, none of the TTV-infected children demonstrated elevated levels of ALT or clinical signs of liver disease. However, this finding does not exclude the possibility that liver disease may develop later in these children. Thus, prospective clinical studies are required to determine the long-term outcome of TTV infection.

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